Absence of Detectable Calmodulin in Cows' Milk by a Modified Gel Electrophoresis Method

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ABSTRACT

With a simple, sensitive, and rapid SDS-PAGE procedure, developed during the course of this study, calmodulin was not found in cows' milk. The absence of calmodulin at the level of detection provided by this procedure (.4 μg) was unexpected. Because milk is a repository for many protein and enzyme systems, and because the mammary gland is known to contain calmodulin, it was conjectured that at least some "leakage" of the protein into milk would occur, especially into colostrum.

INTRODUCTION

Cows' milk (30 m*M* Ca^{2+}) is an excellent source of calcium-binding proteins, including the caseins (8, 11), β-lactoglobulin (β-Lg) (18), α-lactalbumin (α-La) (3, 10), and perhaps others. In our studies concerning calcium-binding proteins in plant systems, we sought an additional reference source of calmodulin (CaM, MW = 17,000 daltons) to those normally used, namely, bovine brain or bovine testes. We conjectured (16) that cows' milk or colostrum might be sources, although Riss and Baumrucker (13) failed to detect CaM in milk using the phosphodiesterase (PDE) method of analysis for this protein. Although they unequivocally detected CaM in mammary tissue in the range of 24 to 51 mg/kg of wet tissue, the PDE procedure might not detect the protein at low levels or its detection might be affected by naturally occurring inhibitors (13). However, cells normally occurring in milk would be expected to provide enough CaM for detection. To detect the protein, therefore, would require the separation of the milk proteins into the casein fraction and the whey protein subfractions, β-Lg and α-La. Subsequently, it would be necessary to further purify the protein fractions by standard chromatographic procedures in an attempt to isolate CaM. The purpose of this study, then, was to isolate and purify CaM, if its presence could be detected, from either milk or colostrum. During the course of this study, we developed a procedure for SDS-PAGE, which is similar to but simpler than other published procedures.

MATERIALS AND METHODS

Sources of Colostrum and Milk

Four liters of fresh colostrum were collected from a Holstein cow from The Pennsylvania State University herd and shipped frozen. The frozen colostrum was maintained at −30°C until use. Four liters of fresh raw milk from a small herd of 100 cows were obtained from a local health food store.

Milk Protein Fractionations

Both colostrum and raw milk were centrifuged at 5000 × *g* for 15 min at 4°C. The fat plug was removed and the skim milk decanted. The procedure of Aschaffenburg and Drewry (1) was used to separate the caseins (which with colostrum were discarded) from the crude α-La and β-Lg fractions. No further attempts were made to purify these proteins, although such purifications are easily accomplished by either Sephadex G100 or DEAE-cellulose chromatography. However, the crude α-La fraction contained electrophoretically distinct components that migrated faster than β-Lg at pH 8.3. These were isolated by DE-52 cellulose chromatography according to the method of Schreiber et al. (15), dialyzed free of salt, and lyophilized.

Received March 24, 1986.
Accepted February 20, 1987.
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In the procedure described, the casein was discarded. In the event that CaM was bound to casein, it would have been inadvertently lost. To check this possibility, raw milk was fractionated by the procedure of Aschaffenburg and Drewry (1). Instead of being discarded, the casein was exhaustively dialyzed against distilled water to remove Na_2SO_4. The dialyzed sample was warmed to 20°C and the pH adjusted to 4.6 to precipitate the caseins. The supernatant was dialyzed and lyophilized, whereas the precipitated casein was dissolved by titration to pH 7.0 with 1 N NaOH and lyophilized. The remaining Na_2SO_4 whey was fractionated into crude α-La and crude β-Lg, dialyzed, and lyophilized as described previously.

Polyacrylamide Gel Electrophoresis

The electrophoresis method described herein for SDS-PAGE is a modification of the basic method of Laemmli (12). Polyacrylamide (12%) gels were prepared from the following solutions: 1) 48 ml 1 N HCl, 36.6 g Tris-base, .23 ml N*,N*,N′,N′ tetramethylethylenediamine dissolved to 100 ml with water and adjusted to pH 8.9 with concentrated HCl, 2) 48 g acrylamide, and 1.26 g N,N′-methylene-bis-acrylamide dissolved to 150 ml with water and filtered, and 3) .14 g ammonium persulfate in 100 ml water. Solutions 1, 2, and 3 were mixed in proportions of 1:3:4, respectively. The electrode buffer was composed of .6 g Tris-base and 2.88 g glycine dissolved in 1 L at pH 8.3. One to 2 mg of protein were dissolved in 50 μl of this buffer along with 10 μl .25% bromophenol blue and 2 drops glycerol. Approximately 100 μg protein was applied per slot. An E-C Vertical Slab Gel Apparatus (either Model EC 470 or Model EC 490),[2] 3-mm slab thickness, was used. Voltage was initially applied at 100 V and increased in consecutive steps of 50 V/10 min to 300 V. Total running time was 3.5 to 4 h. Protein was stained with a solution consisting of .625 g Coomassie blue, 23 ml acetic acid, 11.4 ml methanol, and 216 ml water for 30 to 60 min. Destaining was accomplished with a solution 7% in acetic acid and 10% in methanol or by electrolytic destaining with an E-C unit.

Sodium dodecyl sulfate gel electrophoresis (12% acrylamide) was performed using the exact buffers described except that .3 g SDS/L was added to the electrode buffer. In addition, no stacking gel was used in either the presence or absence of SDS. Protein samples were solubilized with the SDS solvent system described by Basch et al. (2). Approximately 100 μg protein was applied per slot and voltage applied as described for native gels. Running time was approximately 3.5 h, after which the gel was stained and destained as has been described. Detectable (visible) levels of CaM by the SDS method described were determined as follows. A four slot gel was run with protein concentrations of 40.2 ng, .4 μg, 3.125 μg, and 6.25 μg/slot, respectively. The lowest detectable level of CaM was .4 μg/slot.

Stains-All

The procedure for specific staining of calcium-binding proteins on SDS gels using Stains-All, a cationic carbocyanine dye, as described by Campbell et al. (5), was used in this study. This dye is generally regarded as specific for most calcium-binding proteins including calmodulin and the caseins. It is imperative when this stain is used that all SDS be washed from the gel with several rinses of 25% isopropanol.

Amino Acid Analyses

Twenty-four hour acid hydrolysates (6 *M* HCl) of the proteins were analyzed in duplicate using a Beckman 119 CL amino acid analyzer.[2] [^{6}N]Trimethyllysine was identified by comparison of retention time with a standard.

RESULTS AND DISCUSSION

Numerous SDS-PAGE procedures have been reported in the literature, one of the most recent of which was reported by Basch et al. (2). The procedure described herein incorporates many of the essential features of other researchers, namely, Tris-glycine and SDS in the electrode buffer. However, in our procedure (Figures 1 and 2) we used no stacking gel and only 12% acrylamide in the gel slab. Further, the running time is 3.5 h or less vs. running times as long as 18 h for many procedures. A

[2] Reference to brand or firm name does not constitute endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

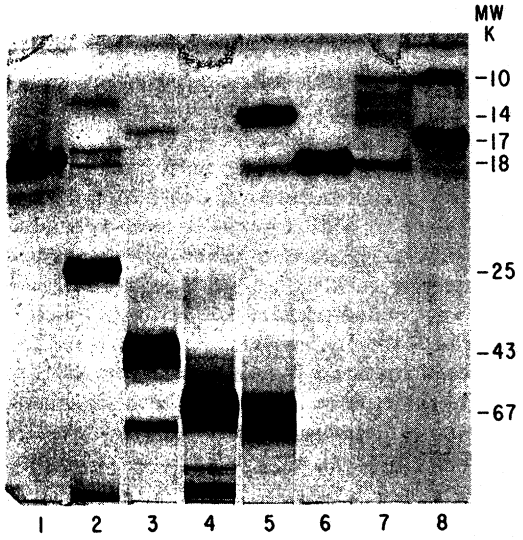

Figure 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of various proteins and fractions. 1, ribonuclease A; 2, chymotrypsinogen A; 3, ovalbumin; 4, serum albumin; 5, α-lactalbumin (crude); 6, β-lactoglobulin; 7, DE-52 fraction (see text); 8, calmodulin contaminated with S-100 protein (10K).

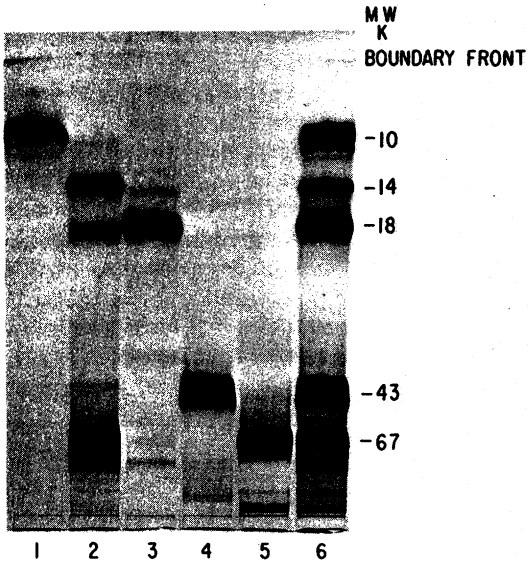

Figure 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of various proteins and fractions. 1, insulin; 2, α-lactalbumin (crude); 3, β-lactoglobulin; 4, ovalbumin; 5, serum albumin; 6, an equal mixture of 1–5 (see text).

complete run, including staining and electrolytic destaining, can be completed within an 8 h period.

Figures 1 and 2 show the resolution of protein zones achieved by the procedure. There are two notable exceptions to a correct molecular weight for proteins applied (Figure 3). The first, ribonuclease A, Figure 1, lane 1, gives an apparent molecular weight of 18K instead of approximately 14K. The second, insulin, Figure 2, lane 1, appears as a dimer of 10K. It was considered that binding of SDS might not have been complete with both of these proteins. After additional heating in SDS and mercaptoethanol, the apparent molecular weight remained the same. Insulin from another supplier also showed a 10K molecular weight. All of the other proteins, however, showed the correct apparent molecular weight when log molecular weight was plotted against relative mobility (Figure 3).

Based upon electrophoretic examinations of whey and casein fractions, described under Materials and Methods, we have never observed a protein zone(s) that could be ascribed to CaM with either native (not reported) or SDS gels. The detectible (visible) level of CaM by the PAGE method described herein is about .4 μg, whereas the PDE method will detect levels as low as .01 μg of pure CaM (4). Further, we have

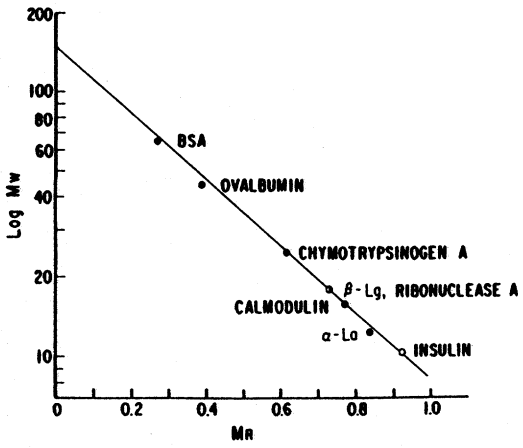

Figure 3. Plot of molecular weight (MW) of protein standards vs. relative electrophoretic mobility (M_r). All data points are measured. Ribonuclease A and insulin do not migrate according to their true molecular weights (see text).

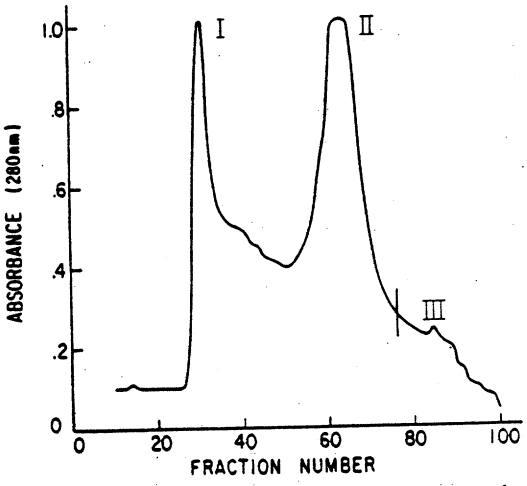

Figure 4. Sephadex G-100 chromatography of a DE-52 cellulose fraction: 160 mg applied in 5 ml to a 2 × 2 cm column in .01 *M* imidazole-HCl buffer. Elution rate: 12 ml/h.

attempted the isolation of CaM from colostrum using an ammonium sulfate fractionation procedure. No detectable CaM was observed upon electrophoresis of the 70% $(NH_4)_2SO_4$ insoluble fraction where CaM usually precipitates.

The only whey protein fraction that stained blue with Stains-All (a feature of calcium-binding proteins) was one recovered from DE-52 cellulose at .30 to .35 *M* NaCl in the salt gradient (CaM also elutes at this salt concentration). It consisted of bovine serum albumin (BSA), β-Lg, and lower molecular weight proteins, less than 14K, which stained blue. Bovine serum albumin and β-Lg were identified by SDS-PAGE. Upon chromatography of this fraction on G-100 Sephadex (Figure 4), a fraction termed III was obtained, examined by SDS-PAGE (Figure 1, lane 7), and found to consist of five proteins. The amino acid analysis of this fraction is shown in Table 1. Notable was the

TABLE 1. Amino acid analyses[1,2] of calmodulin (CaM), β-lactoglobulin (β-Lg), and protein fractions described in text.

Amino acid[3]	Residues/molecule				
	CaM[4]	CaM[5]	Fraction III[6]	Fraction IIIA[6]	β-Lg[4]
Lys	7	8	13	12	15
TML	1	1	0	0	0
Hist	1	1	3	3	2
Arg	6	6	5	3	3
Asp	23	19	14	13	15
Thr	12	12	7	7	8
Ser	4	4	11	8	7
Glu	27	24	33	22	25
Pro	2	3	8	8	8
Gly	11	11	4	5	4
Ala	11	11	7	12	15
1/2 Cys	0	0	2	2	5
Val	7	7	8	9	9
Meth	9	5	2	1	4
Ileu	8	8	11	9	10
Leu	9	9	15	16	22
Tyr	2	2	3	4	4
Phe	8	8	5–6	4	4

[1] 24 h hydrolysates, run in duplicate.

[2] Tryptophan not determined.

[3] Lys = Lysine, TML = trimethyllysine, Hist = histidine, Arg = arginine, Asp = aspartic acid, Thr = threonine, Ser = serine, Glu = glutamic acid, Pro = proline, Gly = glycine, Ala = alanine, 1/2 Cys = cystine, Val = valine, Meth = methionine, Ileu = isoleucine, Leu = leucine, Tyr = tyrosine, Phe = phenylalanine.

[4] Residue numbers from primary structure [references (17) for CaM and (7) for β-Lg].

[5] Calmodulin obtained from Sigma.

[6] See text.

absence of trimethyllysine, an amino acid peculiar to CaM (6), and the inability of this fraction to stimulate phosphodiesterase, another property of CaM (6). Fraction III was rechromatographed on G-100 Sephadex to determine if one of the stronger staining proteins in Figure 1, lane 7, was β-Lg. The amino acid analysis of this fraction, Table 1, IIIA, indicates that the impure fraction closely resembles β-Lg. We have concluded that the remaining low molecular weight proteins from G-100 chromatography represent either fragments of casein or unidentified whey proteins and that further purification is unwarranted. Fraction II obtained from the DE-52 cellulose column was primarily α-La and contained no CaM.

CONCLUSIONS

Based upon the fractionation of two colostrum samples and one whole milk sample and subsequent electrophoretic examination, we conclude that CaM probably does not occur in cow's milk in recoverable amounts. Although CaM was not observed in the whey protein or casein fractions, it might be associated with fat globule membrane proteins. However, it is generally a cytoplasmic protein (13). We have isolated CaM from bovine mammary gland (unpublished results) in high purity, which confirms that it is indeed present in mammary tissue (13, 14). Although Riss and Baumrucker (13) showed the presence of CaM in bovine mammary tissue, they, too, could find no CaM activity in cows' milk, which is peculiar since over 40 enzymes both cytoplasmic and membrane associated have been reported to occur in milks (11). Further, Hira et al. (9) reported the presence of low levels (9.1 μg/L) of CaM in human milk using radioimmunoassay methods. If we assume that bovine milk contained a similar level of CaM and recovery was good by the isolation procedures described herein, the protein would have been detected by SDS-PAGE or by the PDE method.

ACKNOWLEDGMENTS

The authors thank Paul S. Dimick, The Pennsylvania State University, for providing colostrum, and Harold J. Dower, Eastern Regional Research Center, for performing amino acid analyses. We also thank J. Robert Brunner, Michigan State University, for helpful comments and suggestions on this manuscript.

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